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Calcium-activated, phospholipid-dependent protein kinase in cultured rat mesangial cells

Yoshimasa Orita, Yoshihiro Fujiwara, Satoshi Ochi, Yoshimu Tanaka and Takenobu Kamada

First Department of Medicine, Osaka University Medical School, 1-1-50 Fukushima, Fukushima-ku, Osaka 553, Japan

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The analysis of the 100000 × *g* supernatant fraction of cultured rat glomerular mesangial cells with DEAE-cellulose ion-exchange chromatography revealed a large peak showing the activity of a protein kinase (protein kinase C) which depended on phospholipid and diolein as well as Ca^{2+}. Furthermore, it was shown that angiotensin II (AII) (10^{-6} M) induced rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to production of diacylglycerol rich in arachidonic acid, in the cultured rat mesangial cells. These results suggest that activation of protein kinase C resulting from enhancement of phosphoinositide metabolism may be important as an intracellular regulatory mechanism of AII upon cultured mesangial cells.

(Mesangial cell) *Angiotensin II* *Protein kinase C* *Phosphoinositide metabolism* *Second messenger*

1. INTRODUCTION

The glomerula mesangial cell is a target for AII [1]. The contraction and prostaglandin production by AII are important in the regulation of glomerular ultrafiltration process. However, the intracellular mechanism of AII actions on these cells has not yet been clarified and cyclic nucleotides do not appear to play important roles [2]. Protein kinase C, a novel type of protein kinase, whose activity depends not only on Ca^{2+} but also on phospholipid and unsaturated DG, one of the products of phosphoinositide breakdown, has attracted attention as a key enzyme in the regulation of various cell functions [3].

Using cultured rat mesangial cells, this experiment was designed to examine the presence of protein kinase C and a possible relation of enhanced turnover of inositol phospholipids by AII.

Abbreviations: AII, angiotensin II; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol

2. MATERIALS AND METHODS

A mixture of phospholipid, calf thymus H1 histone and [γ-^{32}P]ATP were prepared as in [4–6]. Synthetic diolein was purchased from Nakarai Chemicals. Type I collagenase and soybean trypsin inhibitor were obtained from Sigma. AII (synthetic, human form) was obtained from Peptide Institute, Protein Research Foundation. DEAE-cellulose (DE-52) was obtained from Whatman. Carrier-free [^{32}P]orthophosphate was obtained from Japan Atomic Energy Research Institute. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid was obtained from Amersham.

2.1. *Culture and preparation of rat mesangial cells*

The culture of glomerular mesangial cells was performed as in [7] with some modifications. Briefly, glomeruli were isolated from male Sprague-Dawley rats (70–90 g body wt) by consecutive sieving with 3 different meshes (120, 280 and 53 μm). The glomeruli were cultured at 37°C in a 95% air and 5% CO_2 environment, using the following tissue culture medium: 5 ml RPMI 1640 medium supplemented with 20% decomplemented fetal calf serum (Irvine Scientific) and 1 μg/ml in-

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sulin. Mesangial growth predominated by day 21 of the primary culture when the subculture was performed. The second subcultured cells were employed for this experiment to confirm the following criteria which have already been reported [2,8–13]: (i) the cells had large bundles of intracellular microfilaments; (ii) the intracellular fibrils of the cells were strongly stained by FITC-labelled heavy meromyosin, indicating the abundance of actin; (iii) the intracellular fibrils, cell membranes and extracellular materials of the cells were strongly stained by FITC-antifibronectin antibody; (iv) unlike monocytes, the cells did not show binding capacities for Fc or C3b and phagocytic activities for horseradish peroxidase or sensitized sheep red blood cells; (v) the cells had receptors for AII; (vi) the cells contracted in response to AII. The cells were treated with collagenase and trypsin inhibitor as in [13], and then the cell suspension was prepared by gentle pipetting.

2.2. *Assay for protein kinase C*

Protein kinase C was assayed by measuring the ^{32}P incorporation from [γ-^{32}P]ATP into calf thymus H1 histone in the presence of Ca^{2+}, phospholipid and diolein. The complete reaction mixture (0.25 ml) contained 5 μmol Tris-HCl at pH 7.5, 1.25 μmol magnesium acetate, 50 μg H1 histone, 2.5 nmol [γ-^{32}P]ATP (1.2×10^5 cpm/nmol), 0.25 μmol $CaCl_2$, 20 μg phospholipid, 0.6 μg diolein and enzyme solution. Incubation was carried out for 5 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid and acid-precipitable radioactivity was determined as in [3]. The protein concentration was determined as in [14] with bovine serum albumin as a standard.

2.3. *Assay for phospholipid labelling stimulated by AII*

Cultured cell medium was replaced in Hepes-buffered (30 mM, pH 7.4) Hanks' solution. For [^{3}H]arachidonic acid labelling, the cells were preincubated in Hepes-buffered Hanks' solution containing 0.2% fatty acid-free bovine serum albumin (BSA) and [^{3}H]arachidonic acid (about 2.5 μCi/0.5 ml per 5×10^5 cells). After 120 min preincubation, the cells were washed 3 times with the previous buffer. 10 min after labelling, the experiments were carried out. The cells were incubated for each indicated time (0–1 min) with AII (10^{-6} M). Phospholipids were extracted as in [15]. Separation was done by silica gel thin-layer chromatography as in [16,17]. The spots corresponding to phospholipids such as PIP_2, PIP, PI and DG were scraped into vials and dissolved 10 ml ACS-II (Amersham). The radioactivity was determined using a liquid scintillation counter (Aloca 713).

3. RESULTS AND DISCUSSION

When the crude supernatant was fractionated on DEAE-cellulose ion-exchange chromatography, a large asymmetrical peak showing the enzyme activity appeared in fractions 7–11 (fig.1). Since the

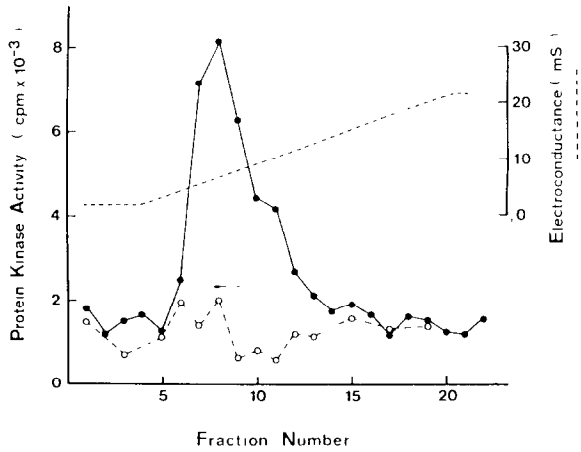

Fig.1. Analysis of protein kinase C in cultured mesangial cells on DEAE-cellulose ion-exchange chromatography. About 1.8×10^6 cells were disrupted by sonication with a Kontes model K881440 sonifier, for 2 min at 4°C in 1 ml of 20 mM Tris-HCl buffer at pH 7.5, containing 0.25 M sucrose, 2 mM EDTA and 5 mM EGTA. The sonicate was centrifuged for 60 min at 100000 × *g* and the supernatant utilized for experiments. An aliquot (0.5 ml) of the 100000 × *g* supernatant was applied to a DE-52 column equilibrated with 20 mM Tris-HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM EGTA. After the column was washed with 4 ml equilibrium buffer, protein kinase C was eluted by a 20 ml linear concentration gradient of NaCl (0–0.3 M) in equilibrium buffer. Fractions of 0.38 ml each were collected. A 50 μl aliquot of each fraction was employed for protein kinase C assay: (●——●) with Ca^{2+}, phospholipid and diolein; (○---○) with 0.5 mM EGTA; (– – –) electroconductance (mS).

protein kinase activity of peak fraction 8 depended on the simultaneous presence of 3 cofactors, that were Ca^{2+}, phospholipid and diolein (table 1), this peak fraction was identified as protein kinase C.

DG, which is derived from the breakdown of phosphoinositides, is the most important modulator of protein kinase C activity [18,19]. Recently, Schlondorff et al. [20] showed that AII caused a decrease of [^{14}C]arachidonic acid-labelled PI and an increase of [^{14}C]arachidonic acid-labelled DG at 2 or 5 min in cultured rat mesangial cells. However, fig.2 shows a decrease of [^{3}H]arachidonic acid-labelled PIP_2 and an increase of [^{3}H]arachidonic acid-labelled at 30 or 60 s of incubation with AII (10^{-6} M). The data in fig.2 indicate that PIP_2 is rapidly hydrolysed within 30 s with a resultant production of DG rich in arachidonic acid, an activator of protein kinase C, in response to AII (10^{-6} M) addition in cultured mesangial cells. The change of [^{3}H]arachidonic acid-labelled PI was hardly shown within 1 min. Fig.3 shows the dose-related effect of AII on DG production after 30 s incubation. In addition, we observed that AII (10^{-6} M) stimulated ^{32}P incorporation into PA and PI but not into PC and PE (0–30 min) (not shown). These results indicate that AII enhances phosphoinositide turnover, primarily by inducing PIP_2 hydrolysis, leading to the formation of DG, in cultured rat mesangial cells.

The formation of DG derived from the breakdown of phosphoinositides with a resultant activation of protein kinase C, leading to phosphorylation of 40 kDa protein, is a key step in the thrombin-induced release of serotonin in human platelets [21–23]. In this experiment, however, we have not yet determined the substrate for protein kinase C which coupled with the activation of this enzyme to the biological function in mesangial cells. A recent report has shown that myosin light chain which, when phosphorylated, regulates the contraction of smooth muscle and non-smooth muscle cells, serves as a substrate for protein kinase C [24] as well as for Ca^{2+}·calmodulin-dependent myosin light chain kinase [25]. Therefore, phosphorylation of myosin light chain by protein kinase C may regulate mesangial cell contraction induced by AII.

An initial event of enhancing turnover of inositol phospholipids by AII appears to be responsible for protein kinase C activation in mesangial cells and a regulatory role of this activation in AII actions via this route is suggestive.

Table 1
Activation of protein kinase C of cultured mesangial cells by Ca^{2+}, phospholipid and diolein

Assay system	Protein kinase activity (cpm)
Complete system	8760
– Ca^{2+} – phospholipid – diolein + 0.5 mM EGTA	1999
– Ca^{2+} + 0.5 mM EGTA	1279
– phospholipid – diolein	1016

A 50 μl aliquot of fraction 8 in fig.1 was employed for protein kinase assay. For the complete system, 1 mM $CaCl_2$, 20 μg phospholipid and 0.6 μg diolein were employed

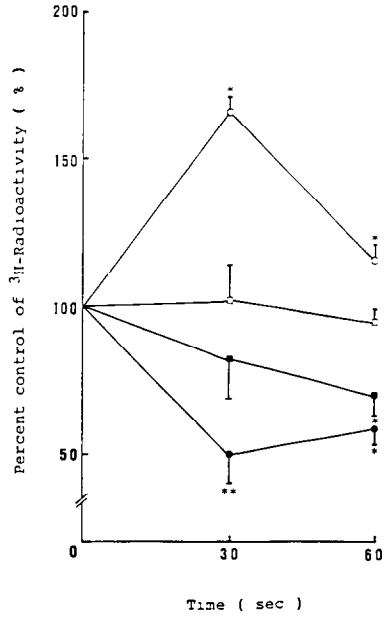

Fig.2. Effect of AII on phosphoinositide breakdown in cultured mesangial cells. Cultured mesangial cells were labelled with [^{3}H]arachidonic acid and then stimulated with AII (10^{-6} M) for the indicated times. (●—●) PIP_2, (■—■) PIP, (□—□) PI, (○—○) DG. The results are means ± SE for triplicate experiments. ** $p <$ 0.01, * $p < 0.05$ vs control.

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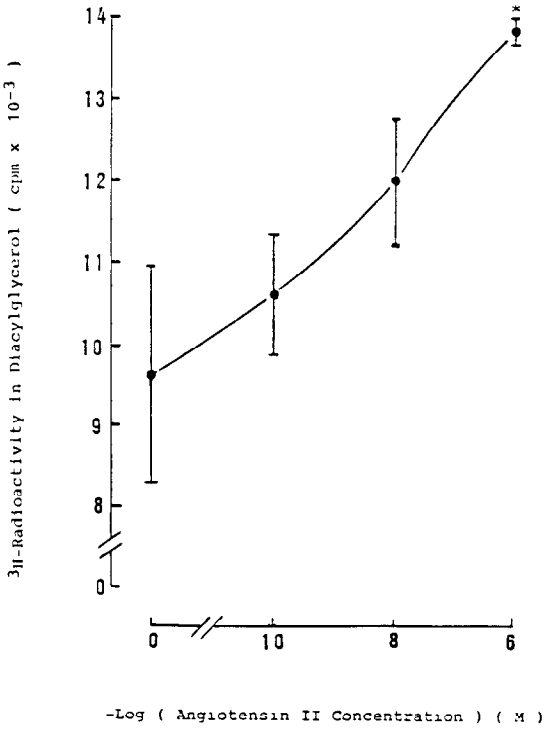

Fig.3. Effect of various concentrations of AII on the formation of DG. Cultured mesangial cells were labelled with [^{3}H]arachidonic acid and then stimulated with AII (10^{-10}, 10^{-8} and 10^{-6} M) for 30 s. The results are means ± SE for triplicate experiments. * $p < 0.05$ vs control.

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